TRANSFORMATION OF POTATO TUBER DISCS USING AGROBACTERIUM

II. POTATO TRANSFORMATION

Materials needed:

Sterile Microtubers
Fresh agrobacterium culture (to be diluted 1:10 in sterile MS below for transformation)
Forceps and scalpel
Vessels for bacterial incubation and subsequent rinse (can be same vessel)
Liquid MS **pH 5.4** (sterilized and stored in 50-100ml alloquots up to12 months)
Sterile water
A sterile surface to cut tubers on (large petri dish)
Sterile filter paper
Plates (falcon 1029 or 1005) of Co-cultivation media

Grow up Agro:

1. Inoculate 10 ml of LB (+ Gm 20 or +Km 50 [for plasmid selection], + Strep [for Agrobacterium selection]) with transformed Agro. Grow up at 28°C overnight.

2. The following morning, dilute the overnight culture 1:10 into fresh Lb + antibiotics + **100µm acetosyringone (final conc.)**. Grow up culture for approx. 4 hours (should be at log phase).

Infect Potatoes:

1. Dilute fresh culture 1:10 with sterile MS pH 5.4 (depending on agro strain).

2. Remove tuber culture from sterile culture vessels and cut away debris--transfer intact minitubers to a clean cutting dish.

3. Add a small amount of sterile water to one side of the dish. Slice minitubers into 1-2mm thick slices. Push cut slices into water to prevent suberization while cutting remaining material.

4. Transfer cut disks to diluted culture, or pour off water and add diluted culture to cutting dish. Incubate in agro/MS at room temp for 10-15 minute. Occasionally stir or swirl slices while incubating in agro/MS, if desired.

5. Pour off agro solution and replace with sterile water or MS. Swirl slices in water (or MS) for 5 min, then remove slices and blot dry on sterile filter paper.

6. Gently place disks , large side down, on Cocultivation med for 2-3 days (23°C, 16 hr light photoperiod). The largest cut surface of the disk should make full contact with, yet not break the surface of, the media. Disks can be placed fairly densely on plates (0.5 cm apart).

7. After 2-3 days of co-cultivation, a moderate (visible, but not excessive) amount of bacterial growth should be evident on the media around the base of each disk. The surfaces not in contact with media should begin suberizing and drying. Following incubation on Cocult. med. transfer to stage one media.

III. REGENERATION

Day 1: Transfer the tuber slices to Stage 1 medium in Falcon 1005 plates. Disks should be placed 8-10 per plate.

Day 7, 14, 21 Transfer to fresh Stage I medium. Transfer to Stage II if callous growth is excessive. Throughout the first month of transfer, cut shoots that generate from the disk and discard. Most of these first shoots are not transgenic--most are growing from 'eyes'. Grooming and pruning the disk and callus seems to promote better health of subsequent tissue.

Day 28 and beyond: Continue to remove and discard shoots that are not growing from callus. There may not be many shoots at this time.

- Transfer disks to Stage 2 medium as usual. Green callus should begin to grow on the surface in contact with the media, mostly from the expanding cells in the periderm. White callus or callus that is not in contact with media (growing from top of tuber slice) is not likely to produce strong transgenic shoots and may be excised. As callus and shoots develop you may want to transfer the tissue to stage 2 in magenta boxes.

- As shoots start to emerge from the **callus**, cut them off and transfer to Stage 3 medium.
Leave the disks/callus on Stage 2 (or transfer to fresh stage 2 media) to form more shoots. If green callus growth is very abundant and the original tuber tissue begins to look unhealthy, it is O.K. to discard original tissue and culture the callus alone.

- Keep track of which disks/callus the various shoots come from by assigning a number to each disk that forms callus. Each disk/callus may be considered one clone—all shoots from the same callus will be assumed to be the same clone. If callus growth begins in two discrete areas with ample area of non-growth between, the disk can be cut in half and each half may be considered a unique transformation event and, thus, a unique clone. This is up to the discretion of the researcher.

- When shoots grow and form roots on Stage 3, slice the shoot portion into nodal segments with a meristem in each segment and transfer to Stage 3 medium again. This increases the number of shoots and insures that the shoots were real transformants. It is also the time to do a Northern analysis to make absolutely sure that the plants you have are really real transformants. BE EVER VIGILANT! WATCH FOR SIGNS OF BACTERIAL CONTAMINATION.Once you have healthy roots on the stage 3 plants, you can transfer them to vermiculite.

IV. TRANSFERING PLANTS FROM CULTURE TO SOIL

1. Fill an appropriate amount of 2.5 inch pots with medium vermiculite. Completely saturate vermiculite with dilute miracle grow or 1/2 strength MS--it may take a while for the vermiculite to absorb the liquid. The vermiculite can be sterilized, but clean, unused vermiculite will do fine.

2. Gently break up agar around plantlet and remove the plant from media and place in a small dish of dilute miracle grow, water, or 1/2 strength MS. Gently wash and try and remove large pieces of agar from the roots. It is not bad to leave agar on the roots, but too much may promote fungus growth once planted in vermiculite.

3. Next, poke a hole in the vermiculite, place plantlet in hole and gently press vermiculite into hole and around the base of plantlet. Place the pots in trays and/or flats that can be sealed to retain moisture and allow light in. Initially, keep light low and moisture high. Once plant is acclimated (4-7 days), allow more light and air flow (lift lid of tray)-- Beware of desiccation! Vermiculite must be kept moist. Plant up to a bigger pot with commercial potting soil when appropriate--usually 2 plants per large pot works well. Be careful when exposing young plants to full light or intense heat--shielding from bright, direct light may be advised for the first day or two in big pots.

V. MEDIA

Autoclave times are for 500ml. Less media may take less time to autoclave. Autoclaving aliquots > 500ml is not recommended.

MS Salts = Caisson Labs #MSP001 = Sigma #5524 = Phytotechnology Labs #M524
LS Salts = Caisson Labs #LSP001 = Sigma #6899

A. Shoot Multiplication Medium (1 Liter) {Blue dot color code}

LS Salts	4.4 grams
Sucrose	30.0 grams
MSMO vitamin stock	1ml of stock (freezer)
Adjust pH to 5.6	
Phytagel (GelRite)	2.0 grams

Autoclave for 20 minutes. Cool to 55°C.
Dispense to sterile Magenta boxes
Alternatively---melt in microwave, pour into tall boxes, and autoclave filled boxes

B. Tuberization Medium (1 liter) {Salmon dot color code}

LS Salts	4.4 grams
Sucrose	60.0 grams
Kinetin (cytokinin)	2.5 ml of 1mg/ml stock
Adjust pH to 5.6	
Phytagel (GelRite)	2.0 grams

Microwave to melt Phytagel.
Dispense into short boxes.
Autoclave filled boxes for 15 minutes.

C. Stage I Medium (1 Liter) {Yellow dot color code}

MS Salts	4.3 grams
Sucrose	20.0 grams
Adjust pH to 5.6	
Phytagel (Gelrite)	2.0 grams

Autoclave 20 minutes. Cool to 55°C. Move to hood and add.

Carbenicillin	10 ml of 50 mg/ml stock. (500µg/ml)
Kanamycin	2 ml of 50 mg/ml stock (100µg/ml)
Gamborg's Vitamin Stock (freezer)	1.0 ml
Zeatin Riboside (freezer)	1.0 ml of 10mM stock
IAA-Aspartic Acid (freezer)	0.1 ml of 3mM stock

Dispense into Falcon 1005 petri dishes (or short boxes).

D. Stage II Medium (1 Liter) {Orange dot color code}

LS Salts	4.4 grams (contains inositol & thiamine)
Sucrose	20.0 grams
Adjust pH to 5.6	
Add Phytagel (GelRite)	2.0 grams

Autoclave 20 minutes. Cool to 55°C. Move to hood.
Aseptically, add:

Gibberellic Acid	1.0 ml stock (10 mg/ml in EtOH)
Zeatin Riboside (freezer)	1.0 ml 10 mM sterile stock
Kanamycin (freezer)	2 ml of 50 mg/ml sterile stock
Carbenicillin (freezer)	10 ml of 50 mg/ml sterile stock
MSMO Vitamin Stock (freezer)	1ml

(OPTIONAL in emergency, add Cefotaxime as requested by researcher)
Dispense to sterile Magenta boxes with membrane lids..

E. Stage III Medium (1 Liter) Kanamycin Selection {Green dot color code}

LS Salts	4.4 grams
Sucrose	30.0 grams
Adjust pH to 5.6	
Add Phytagel (GelRite)	2.0 grams

Autoclave 20 minutes. Cool to 55°C. Move to hood.

Add MSMO Vitamin Stock (freezer)	1.0 ml
Add Kanamycin	4 ml of 50 mg/ml stock (200 µg/ml final)
Add Carbenicillin	10 ml of 50 mg/ml stock.(500 µg/ml final)

(OPTIONAL in emergency, add Cefotaxime as requested by researcher)
Dispense to sterile Magenta boxes.

F. Co-cultivation Medium (1 Liter) {Yellow dot color code}

MS Salts	4.3 grams
Sucrose	20.0 grams
Adjust pH to 5.4 pH is important for vir induction.	
Phytagel (Gelrite)	2.0 grams

Autoclave 20 minutes. Cool to 55°C. Move to hood and add.

Gamborg's Vitamin Stock (freezer)	1.0 ml...
Zeatin Riboside (freezer)	1.0 ml of 10mM stock
IAA-Aspartic Acid (freezer)	0.1 ml of 3mM stock

Dispense into Falcon 1005 petri dishes (or short boxes).

G. Vitamin and Hormone Stocks: Vitamins can be autoclaved with little degradation.
Hormones should not be autoclaved in media.

1. Gamborg's Vitamin Stock (1000X) mg/100 ml Conc. in medium
   myo-inositol.....10,000.....100 mg/L
   Nicotinic Acid.....100.....1 mg/L
   Pyridoxine HCl.....100.....1 mg/L
   Thiamine HCl.....1,000.....10 mg/L
Filter sterilize after dissolving vitamins. Aliquot to sterile tubes and freeze at -20°C.

2. MSMO Vitamin Stock (1000X) mg/100 ml Conc. in medium
   Nicotinic Acid.....100.....1 mg/L
   Pyridoxine HCl.....100..... 1 mg/L
Filter sterilize after dissolving vitamins. Aliquot to sterile tubes and freeze at -20°C.

3. Zeatin Riboside (freezer) 10mM stock
   50 mg + 14.22 ml 0.1N NaOH.
   Filter sterilize and freeze aliquots at -20°C

4. IAA-Aspartic Acid (freezer) 3mM stock
   0.871 mg/ml 0.5N NaOH.
   Filter sterilize and freeze aliquots at -20°C

5. Kinetin: 1mg/ml stock
   Purchase 1mg/ml liquid from sigma (K3253) and dispense to sterile tubes.
   (Can be autoclaved in media with some loss of activity)

6. Gibberellic Acid: 10mg/ml stock
   Dissolve 10mg/ml in 95-100% Ethanol. Aliquot and store at -20°C

H. Antibiotic stocks:

Filter all water soluble antibiotics w/0.22 micron filters, aseptically transfer to sterile tubes and store at -20°C

	Stock	Conc. in Media
Carbenicillin	50mg/ml	500µg/ml
Kanamycin	50 mg/ml	100 (stg1,2)-200(stg3) µg/ml
Cefotaxime	100mg/ml	200-400µg/ml researcher's discretion
Gentamycin	20mg/ml	20µg/ml

A. Shoot Multiplication Medium (1 Liter) .....250ml.....500ml.....750ml
LS Salts.....4.4 grams.....1.1.....2.2.....3.3
Sucrose.....30.0 grams.....7.5.....15.0.....22.5
MSMO vitamin stock .....1ml of stock (freezer) .....0.25.....0.5.....0.75
Adjust pH to 5.6
Phytagel (GelRite) .....2.0 grams..... 0.5.....1.0.....1.5
Autoclave for 20 minutes. Cool to 55°C.
Dispense to sterile Magenta boxes
Alternatively---melt in microwave, pour into tall boxes, and autoclave filled boxes

B. Tuberization Medium (1 liter) .....250ml.....500ml.....750ml
LS Salts.....4.4 grams.....1.1.....2.2.....3.3
Sucrose.....60.0 grams.....15.0.....30.0.....45.0
Kinetin (cytokinin) .....2.5 ml of 1mg/ml stock.....0.625ml .....1.25ml.....1.875ml
Adjust pH to 5.6
Phytagel (GelRite) .....2.0 grams.....0.5.....1.0.....1.5
Microwave to melt Phytagel.
Dispense into short boxes.
Autoclave filled boxes for 15 minutes.

C. Stage I Medium (1 Liter) .....250ml.....500ml.....750ml
MS Salts.....4.3 grams.....1.08.....2.15.....3.0
Sucrose.....20.0 grams.....5.0.....10.0.....15.0
Adjust pH to 5.6
Phytagel (Gelrite) .....2.0 grams.....0.5.....1.0.....1.5
Autoclave 20 minutes. Cool to 55°C. Move to hood and add.
Carbenicillin.....10 ml of 50 mg/ml stock.....2.5.....5.0.....7.5
Kanamycin.....2 ml of 50 mg/ml stock.....0.5.....1.0.....1.5
Gamborg's Vitamin Stock (freezer).....1.0 ml.....0.25.....0.5.....0.75
Zeatin Riboside (freezer).....1.0 ml of 10mM stock .....0.25.....0.5.....0.75

IAA-Aspartic Acid (freezer).....0.1 ml of 3mM stock.....0.025.....0.05.....0.075
Dispense into Falcon 1005 petri dishes (or short boxes).

D. Stage II Medium (1 Liter).....250ml.....500ml.....750ml
LS Salts.....4.4 grams.....1.1.....2.2.....3.3
Sucrose.....20.0 grams.....5.0.....10.0.....15.0
Adjust pH to 5.6
Add Phytagel (GelRite).....2.0 grams.....0.5.....1.0.....1.5
Autoclave 20 minutes. Cool to 55°C. Move to hood.
Aseptically, add:
Gibberellic Acid.....1.0 ml stock .....0.25.....0.5.....0.75
Zeatin Riboside (freezer).....1.0 ml 10 mM stock.....0.25.....0.5.....0.75
Kanamycin (freezer).....2 ml of 50 mg/ml stock (100µg/ml).....0.5.....1.0.....1.5
Carbenicillin (freezer).....10 ml of 50 mg/ml stock.....2.5.....5.0.....7.5
MSMO Vitamin Stock (freezer).....1ml.....0.25.....0.5.....0.75
(OPTIONAL in emergency, add Cefotaxime as requested by researcher)
Dispense to sterile Magenta boxes with membrane lids.

E. Stage III Medium (1 Liter) Kanamycin Selection.....250ml.....500ml.....750ml
LS Salts.....4.4 grams.....1.1.....2.2.....3.3
Sucrose.....30.0 grams.....7.5.....15.0.....22.5
Adjust pH to 5.6
Add Phytagel (GelRite).....2.0 grams.....0.5.....1.0.....1.5
Autoclave 20 minutes. Cool to 55°C. Move to hood.
Add MSMO Vitamin Mix (freezer).....1.0 ml.....0.25.....0.5.....0.75
Add Kanamycin.....4 ml of 50 mg/ml stock .....1.0.....2.0.....3.0
(200 µg/ml final)
Add Carbenicillin.....10 ml of 50 mg/ml stock.....2.5.....5.0.....7.5
(OPTIONAL in emergency, add Cefotaxime as requested by researcher)
Dispense to sterile Magenta boxes.

C. Co-cultivation medium (1 Liter) .....250ml.....500ml.....750ml
MS Salts.....4.3 grams.....1.08.....2.15.....3.0
Sucrose.....20.0 grams.....5.0.....10.0.....15.0
Adjust pH to 5.4
Phytagel (Gelrite) .....2.0 grams.....0.5.....1.0.....1.5
Autoclave 20 minutes. Cool to 55°C. Move to hood and add.
Gamborg's Vitamin Stock (freezer).....1.0 ml.....0.25.....0.5.....0.75
Zeatin Riboside (freezer).....1.0 ml of 10mM stock .....0.25.....0.5.....0.75
IAA-Aspartic Acid (freezer).....0.1 ml of 3mM stock.....0.025.....0.05.....0.075
Dispense into Falcon 1005 petri dishes (or short boxes).